# AIM – AMLYASE PRODUCTION, PURIFICATION AND CONFIRMATION BY BACTERIA

**Requirements – Starch agar and broth, water sample (Sewage water) or Pure cultures of Bacteria, Erlenmeyer Flasks, sterile test tubes and petri plates, inoculation loop, iodine solution, Bench top high speed centrifuge, Saturated solution of Ammonium Sulphate and Distilled water**

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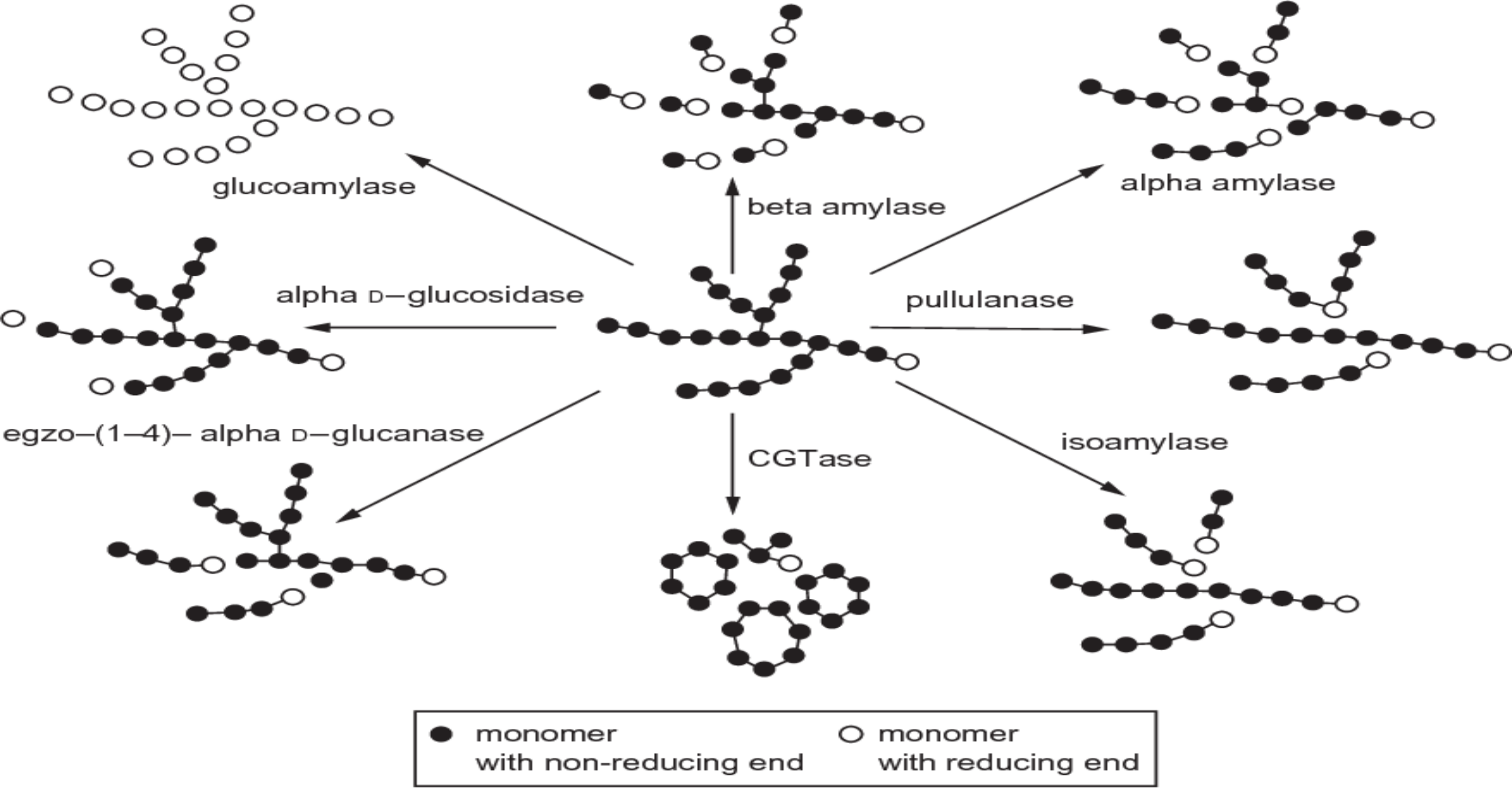
# Principle

- **Amylase is an exoenzyme that hydrolyses starch**
- **Starch is a complex carbohydrate (polysaccharide) composed of two constituents amylase and amylopectin**
- **Amylase production is known in some bacteria like *E.coli and B. subtilis*, while it is well known in fungal species**
- **The ability to degrade starch is used as a criteria for the determination of amylase production by a microbes**
- **In a lab it is tested by performing the starch test in the medium by using iodine solution as an indicator**
- **Starch in the presence of iodine produces a dark blue colour in the area where it is left (Not consumed by the enzyme)**
- **A yellow zone around the growing colony in an otherwise blue surrounding indicates positive amylolytic activity**


glucoamylase
beta amylase
alpha amylase
alpha D–glucosidase
pullulanase
egzo–(1–4)– alpha D–glucanase
CGTase
isoamylase
monomer with non-reducing end
monomer with reducing end

# Procedure

## Primary Screening –

- Prepare Starch Agar medium and pour it into sterile Petri plates and allow to solidify
- Label the each starch agar plates with the name of culture/sample used
- Using sterile techniques make a single streak inoculation of culture/ sample in center of each Petri plate
- Incubate the inoculated plates at 37º C for 24 hrs. in an inverted position
- Flood the surfaces of the plates with iodine solution with the help of dropper for 30 sec.
- Observe for zone of clearance around the growing colony and record the observation as presence or absence of zone of clearance

# Secondary Screening –

- Prepare Starch broth medium and transfer 25 ml of broth to each Erlenmeyer flask
- Inoculate the each Erlenmeyer flask with culture samples
- Incubate the flask at 37º C for 24 hrs./ 2-4 days
- After incubation, centrifuge the culture at 10000 rpm for 15 min.
- Take the supernatant and discard the bacterial pellets
- Pour starch agar into fresh Petri pate, allow to solidify and with the help of sterile cork borer prepare well in the center of the plate
- Pour the supernatant in the wells with the help of Micropipettes (Change the tip of Micropipette for each sample)
- Incubate the plates at 37º C for 24 hrs.
- Flood the plates with iodine solution and observe for zone of clearance around the well
- Record the observation as size of zone (in mm) around the well

# Purification of Enzyme –

- The crude enzyme received after centrifugations needs purification to remove contaminants
- There may be contamination of unspent media ingredients and other soluble compounds which needs to be removed
- Grow the positive and high potential cultures in starch broth (You need a large amount of culture for purification process)
- Centrifuge the culture and collect the supernatant after 2-4 days incubation
- Add saturated solution of Ammonium sulphate to the supernatant for precipitation of proteins, called Salting
- Salting out is a purification method that utilizes the reduced solubility of certain molecules in a solution of very high ionic strength
- Collect the pallet by centrifugation at 10000 rpm for 15 min. (Use cooling centrifuge if available)

# Confirmation of Enzyme Activity –

- **Resuspend the pallet in Distilled water or any suitable buffer**
- **Confirm the enzyme activity by repeating the experiment of Secondary Screening**
- **The pure enzyme may further used for quantitative estimation and electrophoretic separation for characterization of Protein**

# Observation

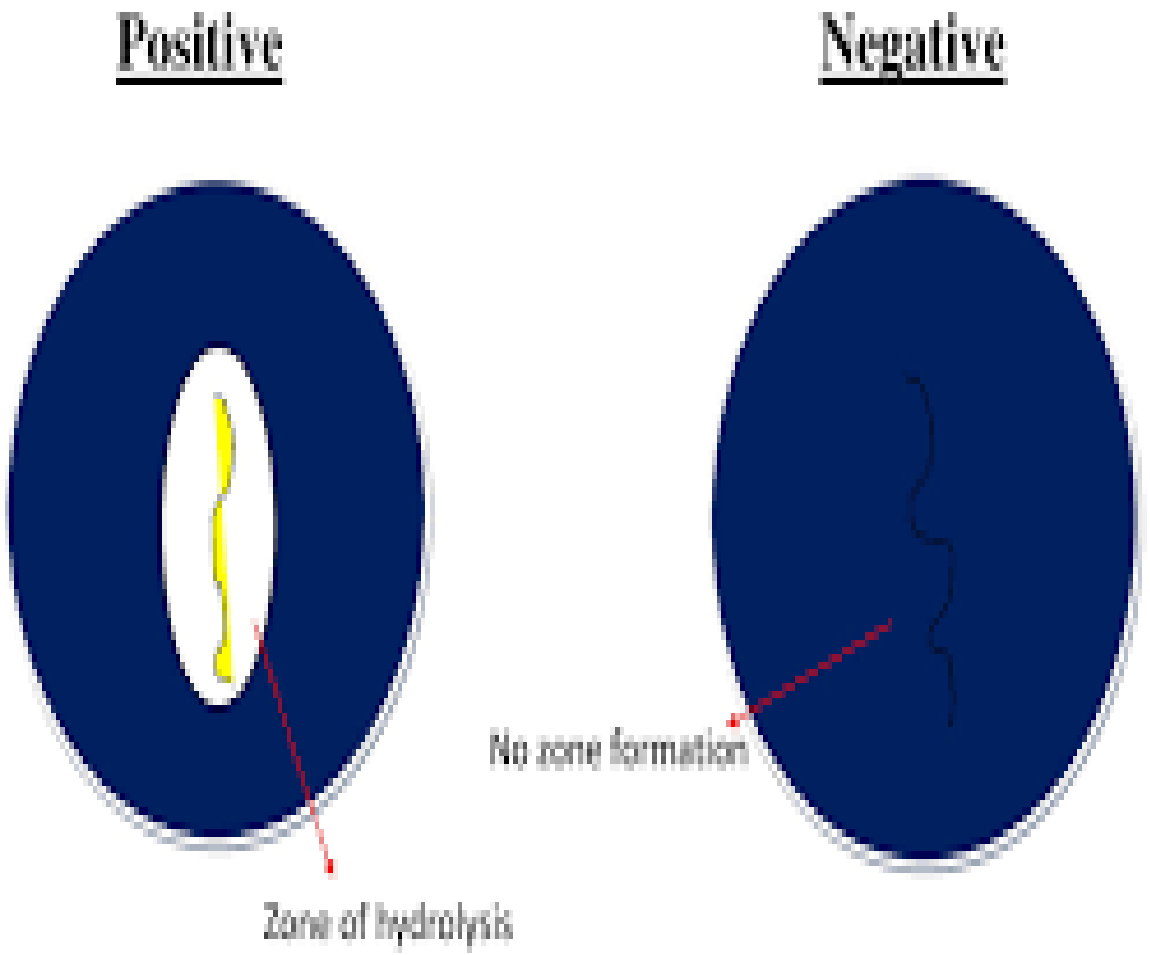
- **Primary Screening:**

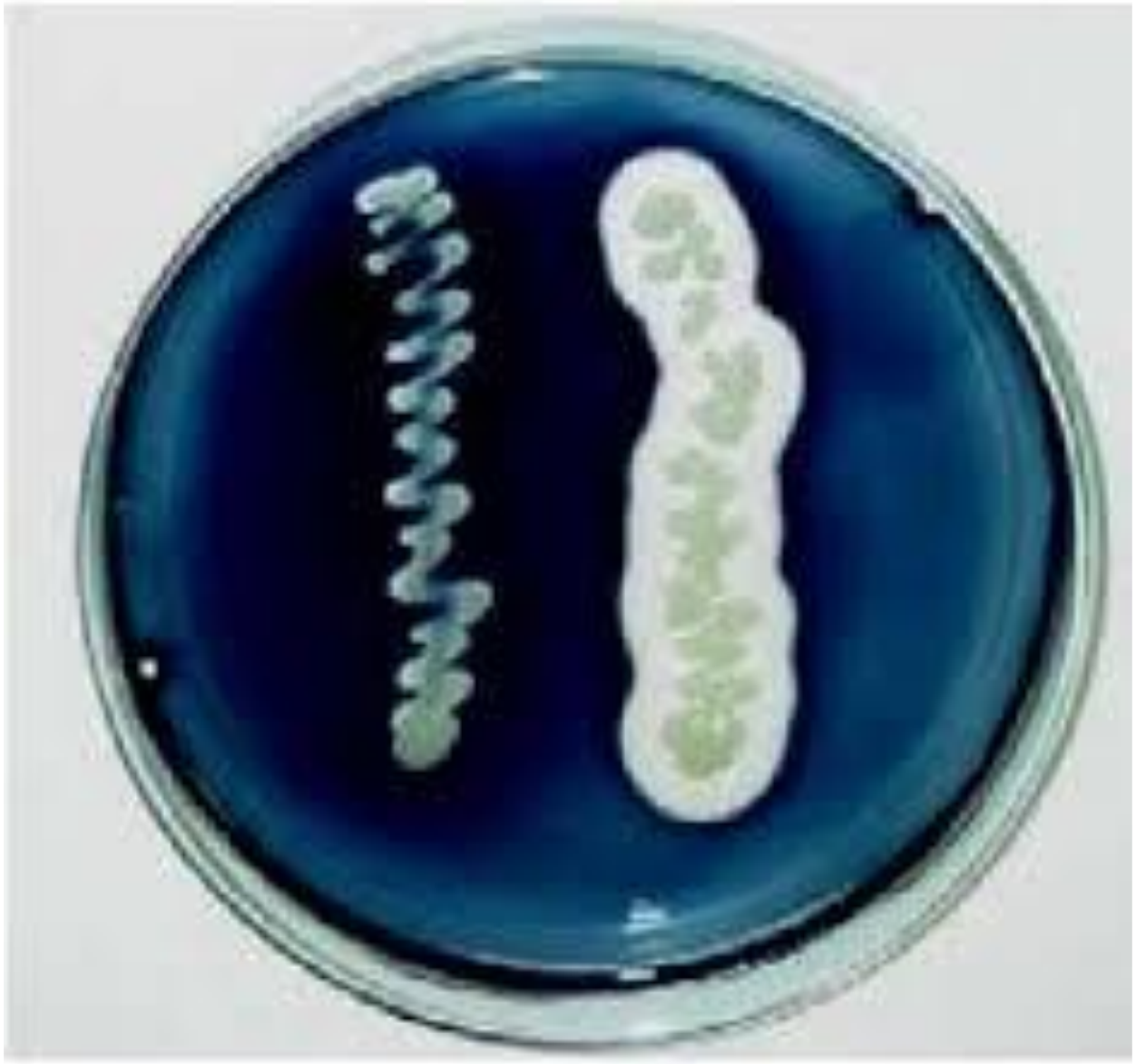
| S.No. | Name/ Code of Culture | Clear Zone * |
|---|---|---|
| | | |
| | | |

*** + Present / – Absent; Observations may be recorded as +/++/+++ or – for low positive, moderate positive or high positive results**

- **Secondary Screening:**

| S.No. | Name / Code of Culture | Size of Clear Zone (mm) |
|---|---|---|
| | | |
| | | |


Positive
Negative
No zone formation
Zone of hydrolysis



## Result – State the result as:

1. Number of positive cultures in primary screening
2. Name of highly positive, moderate positive and least positive cultures
3. Confirmation of amylase production by given culture

## Interpretation –

- Interpret your result as source of microorganism and probability of amylase production

## Precautions –

1. All inoculations should be done carefully
2. Care should be taken while handling the centrifuge
3. All observations should be recorded carefully

# Thank You……